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6. AUTHOR(S)
Alvin Fox

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)
Department of Microbiology and Immunology
University of South Carolina
School of Medicine
Columbia, SC 29208

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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12a. DISTRIBUTION / AVAILABILITY STATEMENT

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12 b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Our biodetection research is primarily concerned with two inter-related issues 1) development of novel schemes for improving chemotaxonomic characterization of microbial pathogens and 2) mass spectrometry-based methods for trace detection of chemical markers for key agents in complex environmental matrices. $180,000 in direct + indirect costs were provided over the period June 1992-1995. This contract was extended with additional funds of $49,020 from ERDEC through June 1995- June 1996. Development of chemotaxonomic schemes for correct identification of species of bacteria relevant to the biodetection program is essential. The research employed a systematic search for new chemical markers employing state-of-the-art chemical and molecular approaches. The feasibility of detecting markers for bacteria in environmental samples was demonstrated by developing a working method for detection of muramic acid (universally found in bacteria) in airborne dust. A prototype approach for the rapid (< 10 minutes) detection/identification of microorganisms (*B. anthracis)* based upon the combination of polymerase chain reaction (PCR) and electrospray mass spectrometry is also described. Extrapolation of this approach to environmental monitoring would represent a major improvement over existing technologies for biodetection.

14. SUBJECT TERMS
Mass spectrometry; bacteria; biodetection

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IDENTIFICATION OF NUCLEIC ACID SEQUENCES AND CHEMICAL MARKERS FOR TAXONOMIC CHARACTERIZATION OF BACTERIA.

FINAL REPORT

A. Fox P.I. and K. Fox Co-P.I.

June 1992-June 1996

Army Research Office

DAAL03-92-G-0255

University of South Carolina
School of Medicine
Department of Microbiology and Immunology
Columbia, SC 29208

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1. FORWARD (optional)

Our biodetection research is primarily concerned with two inter-related issues 1) development of novel schemes for improving chemotaxonomic characterization of microbial pathogens and 2) mass spectrometry-based methods for trace detection of chemical markers for key agents in complex environmental matrices. ARO (contract DAAL03-92-G-0255 provided $180,000 in direct + indirect costs over the period June 1992-1995. This contract was extended with additional funds of $49,020 from ERDEC through June 1995- June 1996. Additional overlapping funds of $160,000 also provided for 1995/1996 (jointly provided by ARO/ERDEC, grant DAAH04-95-1-0359) has contributed to the recent acceleration of this research. Nineteen articles are so far published, in press or submitted. An additional article is currently in preparation.

2. TABLE OF CONTENTS

None

3. LIST OF ALL APPENDICES, ILLUSTRATIONS, AND TABLES (IF APPLICABLE)

None

4. BODY OF THE REPORT

A. Statement of the problem studied

The aim of biodetection is to rapidly detect microbes in an airborne attack. Standard microbiological techniques require days. Development of chemotaxonomic schemes for correct identification of species of bacteria relevant to the biodetection program is essential. These organisms are generally of infrequent occurrence in the medical microbiology laboratory. Often batteries of tests commonly employed are not fully developed for identification of such organisms.

Without accurate identification of organisms, developing biodetection schemes is simply impossible. The research employed a systematic search for new chemical markers employing state-of-the-art chemical and molecular approaches. The feasibility of detecting markers for bacteria in environmental samples was demonstrated by developing a working method for detection of muramic acid (universally found in bacteria) in airborne dust. A prototype approach for the rapid (< 10 minutes) detection/identification of microorganisms based upon the combination of polymerase chain reaction (PCR) and electrospray mass spectrometry is also described. We have demonstrated the feasibility of the approach for characterization of *Bacillus anthracis*, a leading biological agent. Extrapolation of this approach to environmental monitoring would represent a major improvement over existing technologies for biodetection.

B. *Summary of the most important results*

Chemical/molecular schemes for characterization for two of the four leading bacterial species relevant to biodetection have been established - *Bacillus anthracis* and *Brucella melitensis.* In each instance these organisms are extremely difficult to differentiate from closely related species (*B. anthracis* from *B. cereus* and *B. thuringiensis* and *B. melitensis* from *B. abortus* and *B. suis*).

Bacillus anthracis (the causative agent of anthrax) and *B. cereus* (food poisoning) and *B. thuringiensis* (a "natural" pesticide) are closely related genetically. *B. subtilis* is somewhat more distinct. The *B. cereus* group (*B. anthracis*, *B. thuringiensis* and *B. cereus*) and *B. subtilis* were physiologically, molecularly and chemically characterized. The 16S/ 23S rRNA spacer region was amplified giving 3 major products. The PCR products found in strains of *B. subtilis* were approximately 270 ,400, and 430 nucleotides, with the corresponding bands in the *B. cereus* group at 250, 430, and 480. These patterns allowed *B. subtilis* to be readily differentiated from the other 3 species. (Wunschel, Fox, Black and Fox, 1994).

Using GC-MS, sugar profiles of vegetative cells were indistinguishable for *B. cereus* and *B. thuringiensis*. *B. anthracis* contained high levels of galactose which generally distinguished it from *B. cereus/B. thuringiensis*, while *B. subtilis* was distinguished from the *B. cereus* group by low mannosamine levels. Spore profiles differed from vegetative profiles in all 4 species. Like vegetative profiles, spore profiles were distinctive for *B. cereus/B. thuringiensis*, *B. anthracis*, and *B. subtilis*. *B. cereus* and *B. thuringiensis* spores both contained rhamnose, fucose, 2-O-methyl rhamnose and 3-O-methyl rhamnose, unlike *B. anthracis* spores which contained only rhamnose and 3-O-methyl rhamnose. *B. subtilis* strains were heterogeneous with some resembling *B. anthracis* and others *B. cereus/B. thuringiensis*, although *B. subtilis* strains typically contained quinovose. The *B. cereus* group can be easily distinguished from *B. subtilis*, however, differentiation within this group has always been problematic. Using carbohydrate profiling, *B. anthracis* was readily distinguished from *B. cereus/B. thuringiensis*. (Fox, Black, Fox and Rostovtseva, 1993; Wunschel, Fox, Black and Fox, 1994).

Carbohydrate profiles readily differentiated *B. canis* from the other three species of *Brucella* that cause human disease. Each of the 7 strains of *B. canis* was unique in lacking quinovosamine. *B. abortus* (6 strains), *B. melitensis* (4 strains) *and B. suis* (one strain) all contained quinovosamine. Quinovosamine is not commercially available and was identified by GC-MS analysis. It displayed identical gas chromatographic retention time and mass spectrum to quinovosamine previously identified by us in *Legionella pneumophila*. Amplification of the rRNA 16S / 23S spacer region generated 1 band (127 bp) characteristic of the genus *Brucella* but absent in other species of bacteria. These results were presented at the 1996 ASM and a manuscript is in preparation. Unfortunately, *Brucella* require growth in a P3 facility. Thus in order to study these organisms they must first be characterized physiologically. The killed organisms are then sent to USC for study with more advanced chemical and molecular analysis. The logistics of growth of strains has been established. However, further work is needed to allow routine physiological characterization. This is slowing down completion of chemotaxonomic studies.

Although GC-MS profiling of microbial carbohydrates provides a useful universal method for identification of bacteria it is extremely time consuming. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) show great promise for rapid identification of sugar markers in their native form. The potential of this approach has been demonstrated (Black and Fox, 1995). This builds on an earlier work performed in collaboration with Dr. Pete Snyder of the ERDEC using electrospray MS-MS without chromatographic separation (Black, Fox, Fox, Smith, and Snyder, 1994.

There are currently only three chemical markers that are being used in monitoring airborne biocontamination as noted in the "Field Guide for the Determination of Biological Contaminants in Environmental Samples" published by the American Industrial Hygiene Association. Muramic acid which is a universal marker for bacteria. Hydroxy fatty acids as markers for Gram negative bacteria and ergosterol for fungi. All are currently detected by gas chromatography-tandem mass spectrometry (GC-

MS-MS). None of these methods are suitable for military biodetection since they take many hours for dust collection and analysis. However, they prove the validity of the concept of detecting bacteria in air by mass spectrometry based instrumentation. The procedure for detection of muramic acid in air was developed with funds from ARO/ERDEC and (Fox et al. 1993; Fox et al. 1995; Fox, Harrelson, Krahmer, in press). The Center for Indoor Air Research (Jan 1991-Dec 1995) also provided $650,000 (1992-1996) for this project to Dr. Fox and his collaborator (Dr. Lennart Larsson, University of Lund). Dr. Larsson developed the GC-MS-MS procedures for ergosterol and hydroxy fatty acid monitoring mentioned above.

For purposes of biodetection, there is need of an approach that is rapid and can identify to the species level. The polymerase chain reaction (PCR) can be used to detect genes with known sequence composition that are diagnostic of specific microorganisms. These include their virulence genes (including coding for adhesion molecules, exotoxins, or enzymes involved in capsule synthesis) or variable portions of conserved genetic regions (e.g. the ribosomal rRNA genes or the spacer region between rRNA genes). For example, the spacer region is highly variable in length among bacterial species. Thus the molecular weight of the PCR product alone (without sequencing) identifies to the species level. PCR amplification is possible in ~5 minutes, and achievable speed depends upon both the steps in the PCR amplification and the sensitivity of the subsequent detection method (this determines the number of amplification steps required). However, traditional methods for analysis of PCR products have involved gel electrophoresis, which is far too slow for biodetection.

In contrast, rapid and precise analysis of PCR products has been recently achieved by the P.I and his collaborators (Richard Smith, Pacific Northwest National Laboratory) using ESI-MS. Interaction with staff at ERDEC has also helped to focus our preliminary efforts. Development of a compact and integrated methodology for rapid detection and identification of biological agents in environmental matrices using PCR-MS appears realistic. The power of the approach is the use of mass spectrometric analysis that provides rapid detection/identification (<1 minute), a speed that greatly exceeds that of conventional electrophoresis approaches. A total detection/identification time for biological agents in environmental samples of under 10 minutes appears feasible. This work involved the use of a fourier transform ion cyclotron resonance mass spectrometer (FTICR-MS) for analysis of PCR products. More conventional instrumentation based upon ion trap instrumentation may suffice, and the utility of such instrumentation needs to be determined. The achievement of such high-speed analysis with high levels of sensitivity and specificity represents a major improvement over existing technologies for biodetection.

Basic research has resulted in demonstration of feasibility of a new methodology capable of use in rapid detection of biological agents in environmental matrices. The present work employed pure microbial cells for PCR-MS analysis. There is a need for further basic research to prove that molecular markers for specific microbial species and genera can be detected in complex environmental samples using both conventional PCR and PCR-MS. Collaboration with ERDEC and other components of DOD will be required in applied research to engineer a final instrumental product suitable for biodetection.

C. *List of all publications and technical reports*

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12. Black G. and Fox, A. Recent progress in analysis of sugar monomers from complex matrices using chromatography (GC/LC) in conjunction with mass spectrometry (MS, MS-MS) or stand-alone MS-MS. J. Chromatogr. 720: 51-60. 1996.
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15. D. Wunschel, K. Fox, A. Fox, J. Bruce, D. Muddiman and Smith R. Analysis of double stranded polymerase chain amplification products from the *B.cereus* group by electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Spectrom. 10: 29-35. 1996.
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20. K. Kim, T. Hadfield, K. Fox and A. Fox. Differentiation of *Brucella* species using PCR and gas chromatography-mass spectrometry. Presented at the American Society for Microbiology. Annual Meeting. May, 1996. **Manuscript in preparation**

D. *List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project*

Gavin Black received his Ph.D. May 1995. He was awarded an NRC fellowship to work at ERDEC under the supervision of Peter Snyder. David Wunschel is expected to graduate Dec 1996. He will be supervised by Richard Smith (PNRL) and is expected to continue working in areas related to biodetection (characterization of nucleic acid using mass spectrometry and capillary electrophoresis)

5.REPORT OF INVENTION

Automated evaporator for chemical analysis. Patent number 5514336. Issued May 7 1996.

6. BIBLIOGRAPHY

See above

7. APPENDICES

None